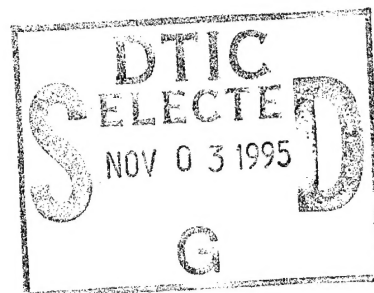


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The Latency of Exocytosis Varies with the Mechanism of Stimulated  
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by

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# The Latency of Exocytosis Varies with the Mechanism of Stimulated Release in PC12 Cells

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*Abbreviations used:* ACh, acetylcholine; DA, dopamine; IP<sub>3</sub>, inositol trisphosphate;

NA, norepinephrine; SSCE, sodium saturated calomel electrodes; VSCC, voltage-

sensitive calcium channels.

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# **ABSTRACT**

To compare the time course of different mechanisms of chemically stimulated release, amperometric detection of dopamine was carried out at single PC12 cells. The rapid response of carbon fiber microelectrodes allowed the detection of single exocytotic events thus providing time-resolved information about the dynamics of stimulated release, in particular the latency between the stimulation of a cell and the secretion of catecholamines. Upon rapid depolarization of the cell membrane caused by application of 105 mM K<sup>+</sup>, almost immediate ( $6\pm 1$  s) release of dopamine was observed. Stimulation with 1 mM nicotine, involving the stimulant binding to a ligand-gated ion channel, resulted in a short ( $37\pm 5$  s) delay between stimulation and secretion. The application of 1 mM muscarine to the cells caused a long ( $103\pm 11$  s) latency before exocytosis was detected. A biphasic response that appeared to be similar to a combination of nicotine- and muscarine-stimulated release was observed when cells were stimulated with 10 mM acetylcholine. Thus it appears that the dynamics of stimulated release at single PC12 cells is significantly affected by the mechanism leading to exocytosis.

## INTRODUCTION

Intercellular communication involving the process of exocytosis has been the focus of much research for several decades (Reichardt and Kelly, 1983; Almers, 1990; Lindau and Gomperts, 1991; Jahn and Südhof, 1994). The fact that exocytosis occurs by the fusion of intracellular vesicles with the plasma membrane (Heuser, 1976; Heuser and Reese, 1979) with subsequent release of vesicular contents has been fairly well established, but the steps leading up to these events are not as clearly understood. In general, intracellular  $\text{Ca}^{2+}$  is required for exocytosis (Ritchie, 1979), though release has been shown to occur in its absence (Pozzan et al., 1984). At sufficient levels in the cytoplasm,  $\text{Ca}^{2+}$  causes the mobilization of vesicles to the plasma membrane where rapid exocytosis follows (Smith and Augustine, 1988).

The entry of  $\text{Ca}^{2+}$  into the cytoplasm and subsequent exocytosis can be accomplished in three basic ways. The first and simplest mechanism is the depolarization of the cell membrane which triggers the influx of extracellular  $\text{Ca}^{2+}$  through voltage-sensitive  $\text{Ca}^{2+}$  channels (VSCC) (Stallcup, 1979). Depolarization may be accomplished by electrical stimulation or by elevated extracellular  $\text{K}^+$  levels. The second mechanism involves the binding of the stimulant to a ligand-gated ion channel such as the nicotinic acetylcholine receptor, which causes the ion channel to open, resulting in depolarization of the membrane and influx of  $\text{Ca}^{2+}$  through VSCC (Stallcup, 1979). The third, most complex and least understood mechanism involves a stimulant binding to a receptor which causes the transduction of intracellular signals

through second messengers leading to the eventual release of  $\text{Ca}^{2+}$  from intracellular stores (Berridge and Irvine, 1984; Stoehr et al., 1986). The muscarinic acetylcholine receptor is an example of this type of receptor.

The PC12 clonal cell line, derived from a pheochromocytoma of the rat adrenal gland (Greene and Tischler, 1976) and commonly used as a model for sympathetic neurons, is used in this study. PC12 cells provide all the components necessary to study elevated  $\text{K}^{+}$ -, nicotine- and muscarine-stimulated release. They synthesize, store, release and take up the catecholamines, dopamine (DA) and norepinephrine (NA) (Greene and Tischler, 1977a). They contain voltage-sensitive  $\text{Na}^{+}$ ,  $\text{K}^{+}$  and  $\text{Ca}^{2+}$  channels (Shafer and Atchison, 1991), receptor-mediated  $\text{Na}^{+}$  and  $\text{Ca}^{2+}$  channels (Stallcup, 1979) and  $\text{Ca}^{2+}$ -sensitive  $\text{K}^{+}$  channels (Pun and Behbehani, 1990). PC12 cells also express muscarinic receptors (Jumblatt and Tischler, 1982; Cross et al., 1984) and nicotinic receptors (Patrick and Stallcup, 1977; Whiting et al., 1987). Upon chemical stimulation, PC12 cells exhibit  $\text{Ca}^{2+}$ -dependent exocytosis (Greene and Rein, 1977b; Baizer and Weiner, 1985; Rabe et al., 1987).

Most studies involving mechanisms of release in PC12 cells have been carried out using radiochemical assays (Vincentini et al., 1986; Rabe et al., 1987; Inoue and Kenimer, 1988). This method requires sampling from a population of cells and does not provide the time resolution needed to follow the dynamics of exocytosis at single cells. In order to obtain individual release event data from single cells, a small, sensitive probe with a rapid response time (ms) is necessary. These parameters are met

with bevelled carbon fiber microelectrodes (Kelly and Wightman, 1986). They allow detection of single exocytotic events at the cellular level both in vivo (Garris et al., 1994) and in vitro (Leszczyszyn et al., 1990; Wightman et al., 1991; Chow et al., 1992; Chen et al., 1994). The selectivity of electrodes for electroactive species provides the specificity required to work in complex biological matrices. The catecholamines, DA and NA, released from PC12 cells are easily oxidized and so are readily detected. Amperometry at these electrodes provides quantitative information regarding the released catecholamines.

In this paper, we show that exocytosis from single PC12 cells occurs with dramatically different time courses following activation of the cells by different mechanisms. The use of bevelled carbon fiber microelectrodes in the amperometric mode at cell surfaces allows the detection of the first release event following cell stimulation as well as the measurement of subsequent events in a time-resolved manner. Not only have the dynamics of elevated  $K^+$ -, nicotine- and muscarine-stimulated release been investigated, but the average vesicular catecholamine content and distribution have been compared. The data presented here shows that the time course of exocytosis is affected by the mode of stimulation.

## METHODS AND MATERIALS

### *Cell Culture and Solutions*

Stock PC12 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in phenol red free RPMI-1640 media supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (JRH Bioscience, Lenexa, KS) and 0.2% antibiotic antimycotic solution in a 7% CO<sub>2</sub>, 100% humidity atmosphere at 37° C, as described by Greene et al. (1991). Cells were grown to near confluence in mouse collagen IV coated (0.5µg/cm<sup>2</sup> in 0.05N HCl, Collaborative Biomedical Products, Bedford, MA) Falcon cell culture dishes (60 mm). The cells were subcultured every 7-10 days at one fourth their original density with the medium being changed every 2-3 days. Cell experiments were performed on passages 1-10. A previous study has shown that maximum DA secretion occurs in the late exponential phase of growth (Takashima and Koike, 1985) which was found to correspond to 5-11 days after subculturing. Therefore all experiments were performed in this time frame. It was observed in previous experiments that no release was normally detected on the day after medium was changed, therefore no cell experiments were run on these days. Prior to carrying out the experiments, the cells were washed twice with 4 mL of warm (37° C) unsupplemented media. All stimulant solutions (1mM (-)-nicotine hydrogen tartrate, 1 mM muscarine chloride, 10 mM acetylcholine (ACh) chloride) were prepared in buffered saline solution (150 mM NaCl, 4.2 mM KCl, 2 mM CaCl<sub>2</sub>, 0.7 mM MgCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub> and 10 mM HEPES). Stimulation



solutions were of supramaximal concentration to maximally activate the receptors and prevent unwanted kinetic effects. The elevated potassium stimulation solution was also prepared in buffered saline solution with 105 mM KCl and 5 mM NaCl. All solutions were prepared fresh daily from stock solutions and contained 0.1% (by wt.) fast green dye as a visual aid.

### *Electrochemical Measurement of Exocytosis*

Carbon fiber microelectrodes were fabricated by aspiration of a 5- $\mu$ m diameter carbon fiber (Amoco, Greenville, SC) into a 1.2 mm X 0.68 mm glass capillary (A-M Systems, Everett, WA). The fiber was sealed in the glass capillary by pulling the glass on a vertical capillary puller (Ealing, Harvard Apparatus, Edenbridge, KY). The tip of the electrode was then dipped into epoxy (Epo-Tek, Epoxy Technology, Billerica, MA) for 30 s and cured at 100°C for 1-2 hrs. The electrodes were back-filled with colloidal graphite (Polysciences, Inc., Warrington, PA). The carbon fiber was trimmed at the glass-fiber junction with a scalpel to expose an unoxidized electroactive surface. The electrode tip ( $\approx 7\mu$ m) was bevelled at approximately 40° on a micropipette beveler (World Precision Instruments, New Haven, CT) with diamond paste (0.25  $\mu$ m diam., Buehler, Lake Bluff, IL) for 2 min. After beveling, the tip was sonicated in a 75% methanol solution for 30 s to remove any adhered diamond paste (Kawagoe et al., 1993). Electrodes were examined under a microscope to be sure the tip was smoothly

bevelled and their response was tested in 0.1 mM DA using slow scan cyclic voltammetry prior to use. Locally constructed sodium saturated calomel electrodes (SSCE) were used as reference electrodes.

Amperometric detection was carried out at 650 mV vs SSCE with an EI-400 potentiostat (Ensmann Instrumentation, Bloomington, IN) in the two-electrode mode. A built-in 2-pole low-pass filter in the EI-400 was set at 100 Hz; no other electronic filtering device was used. Amperometric data points were collected and displayed every 1.22 ms (820 Hz) on a Gateway 2000 386/25 PC computer (with 8 MB RAM memory) with a Labmaster interface (Scientific Solutions, Solon, OH).

All cell experiments were performed on the stage of an inverted microscope (IM-35, Carl Zeiss, Thornwood, NY) at room temperature. The carbon fiber working electrode was gently placed on the target cell with a piezomicropositioner (PCS-750/1000, Burleigh Instruments, Fishers, NY). This arrangement allows site-specific monitoring of the release, measuring a region within 2  $\mu\text{m}$  of the carbon fiber electrode tip (Schroeder et al., 1994). A micropipette (10  $\mu\text{m}$  tip diameter) containing the stimulation solution was positioned approximately 100  $\mu\text{m}$  from the cell of interest using a micropositioner (Zeiss, Germany). The stimulant was administered by a 6 s pressure injection at 6 psi (Picospritzer II, General Valve, Fairfield, NJ). The estimated total injection volume was 30 nL per injection. To eliminate unwanted effects from extracellular catecholamines and cellular recovery time, only data from the first 40 s of

release after the first detected event was used for analysis. Visual observations of the cells were made both prior to and after collection of the data to ensure that the observed release was not due to cell damage incurred from electrode placement. All errors are reported as SEM.

### *Data Treatment*

A locally written program was used to acquire, store, display and analyze data. Voltage output from the potentiostat was first digitized by an A/D converter and then displayed as a current vs. time trace before being stored in memory for later analysis. Peaks representing catecholamine release were identified by using four consecutive data points with a positive slope  $>1.4$  times the slope from 10 different 60 Hz noise waves as the starting point of a peak. The end of a peak was marked by the first data point having a value identical to the initial data point of the peak or the first point of four consecutive data points with a positive slope immediately following a negative slope, whichever of these two was larger. To prevent analysis of nonexocytotic events, only peaks with a baseline width less than 40 ms were used for data analysis. The area under each peak or current transient was determined using a locally written program. This area corresponds to charge in coulombs which can be related to the number of molecules of DA released from a single vesicle (Wightman et al., 1991; Chow et al., 1992) using Faraday's law,  $Q = nNF$ , where  $Q$  is the charge,  $n$  is the number of

electrons transferred per mole of the substance oxidized (2 for DA),  $F$  is the Faraday constant (96,485 coulombs/equivalent) and  $N$  is the total number of moles of DA detected by the electrode.

### *Chemicals*

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

## RESULTS

Clonal PC12 cells grown in culture exist as single spherical cells with a 5-12  $\mu\text{m}$  diameter. Like sympathetic neurons, they contain a higher % of DA than NA due to the absence of exogenous ascorbate, which enhances the synthesis of NA (Greene and Rein, 1978). Upon exposure to various chemical stimulants, PC12 cells release their stored catecholamine by a  $\text{Ca}^{2+}$ -dependent mechanism (Greene and Rein, 1977a&b; Baizer and Weiner, 1985; Rabe et al., 1987; Chen et al., 1994).

Individual exocytotic events are depicted as a temporal spike with a rapid rise and more gradual fall in current as the DA is oxidized at the electrode tip. Application of elevated  $\text{K}^+$  stimulant results in immediate and multiple current transients (Fig. 1A) which cease within  $30 \pm 3$  s. The mean lag time between the stimulant administration

and transmitter release has been observed to be  $6 \pm 1$  s for 17 cells (475 total events). When 1 mM nicotine is administered to the cell, the release of DA is not immediate (Fig. 1B) and continues for  $55 \pm 10$  s following a delayed onset. The average latency between stimulation and secretion for nicotine has been measured at  $37 \pm 5$  s for 16 cells (232 total events). Upon stimulation with 1 mM muscarine, the response is delayed to an even greater extent (Fig. 1C) and lasts a relatively long time ( $133 \pm 20$  s). The mean delay time for release following muscarine stimulation has been calculated to be  $103 \pm 11$  s for 19 cells (439 total events). The distributions of latencies for all events in the first 40 s of release are compared for all three stimulants in Figure 2.

The mean vesicular DA content for the stimulus-secretion events has been determined by evaluating the area under each current transient, which corresponds to the charge detected for the oxidation of the contents of a single vesicle (Wightman et al., 1991; Chow et al., 1992). The average vesicle content for cells exposed to elevated  $K^+$  has been measured as  $199 \pm 14$  zmol, while that for exposure to 1 mM nicotine was observed to be  $182 \pm 9$  zmol. The mean vesicle content for cells stimulated with 1 mM muscarine was  $204 \pm 10$  zmol. Distributions of the vesicular dopamine content for these three stimulants are shown separately in Figure 3. Like the mean vesicle content, the three distributions are similar.

The histograms of vesicle content have a skewed distribution with some current transients having a vesicular catecholamine content that is much larger than the average

value. In order to convert these histograms into a form that provides more insight into the vesicle size distribution, an assumption that the catecholamine concentration within each vesicle remains constant has been made (Wightman et al., 1991). Since concentration is equivalent to moles/volume and  $V = 4/3\pi r^3$ , then  $r$  is proportional to the cube root of the vesicle content. Histograms of the percent of total events vs. vesicle content in  $\text{zmol}^{1/3}$  for the data displayed in Figure 3 are shown in Figure 4. The distributions appear to be roughly Gaussian for all three forms of stimulation. This is consistent with observations in adrenal cells by electron microscopy where a Gaussian distribution of vesicle radii is observed (Coupland, 1968).

In further investigations of the latencies observed for the nicotine- and muscarine-stimulated release involving receptor-mediated mechanisms, 10 mM ACh has been used. ACh is expected to bind to both the nicotinic and muscarinic ACh receptors. In 7 of 12 cells, a two-phase response has been observed (Fig. 5) in which secretion ceases for a short time ( $51 \pm 9$  s) before starting again. The average latency for the first response has been observed to be  $34 \pm 11$  s with a mean duration of  $25 \pm 7$  s for (98 events for the 7 cells), while the mean latency for the second response has been determined to be  $109 \pm 15$  s from the time of stimulation with an average duration of  $103 \pm 33$  s (878 events for the 7 cells). The mean vesicular content for both responses has been measured to be  $247 \pm 35$  zmol (1st response) and  $326 \pm 45$  zmol (2nd response).

The other 5 cells did not respond or had a biphasic response in which the first release was immediate.

## DISCUSSION

The rapid response (ms) of carbon fiber microelectrodes in the amperometric mode has allowed the monitoring of the dynamics of exocytosis at single cells. By resolving single release events, this method provides a straight forward means by which to compare the time course of stimulation-secretion processes triggered by different mechanisms. Using this method of detection it has been clearly shown here that the mechanism of stimulated release does indeed affect the dynamics of exocytosis.

Elevated extracellular  $K^+$  levels are known to depolarize the cell membrane and so trigger exocytosis by opening voltage-sensitive  $Na^+$  channels which causes the subsequent opening of VSCC (Ritchie, 1979; Stallcup, 1979). As shown previously, exocytosis from PC12 cells is  $Ca^{2+}$ -dependent (Chen et al., 1994). The opening of  $Ca^{2+}$  channels allows a rapid increase in the intracellular  $Ca^{2+}$  concentration (100 nM at resting to 300-400 nM following stimulation) (Pozzan et al., 1984) to a level sufficient to trigger the mobilization of catecholamine-containing vesicles to the plasma membrane for exocytosis. Because the application of high  $K^+$  leads to immediate depolarization and  $Ca^{2+}$  entry, exocytosis occurs most rapidly ( $6 \pm 1$  s) by this mechanism as evidenced by the data shown here.

Administration of 1 mM nicotine induces exocytosis by a mechanism involving the binding of the stimulant to a membrane receptor. It has been shown that this receptor changes conformation upon ligand binding which opens a channel permeable to  $\text{Na}^+$  primarily but also to  $\text{Ca}^{2+}$  to a small extent. The influx of  $\text{Na}^+$  causes sufficient depolarization of the membrane to open VSCC (Stallcup, 1979) and so allows a more rapid influx of  $\text{Ca}^{2+}$ . It is assumed that the entry of  $\text{Ca}^{2+}$  through the receptor channel does not occur at a rate sufficient to trigger exocytosis before the VSCC are opened. The additional actions of ligand binding to the receptor and the subsequent receptor channel opening prior to massive  $\text{Ca}^{2+}$  influx accounts for the longer time observed between stimulation and secretion ( $37 \pm 5$  s) upon application of 1 mM nicotine. Although this delay seems long, the most dramatic latencies were observed upon application of 1 mM muscarine.

The mechanism by which muscarine activates release is known to involve the binding of stimulant molecules to a membrane receptor followed by the transduction of signals through intracellular second messengers but beyond this, much is open to further study. PC12 cells contain more than one type of muscarinic receptor (Bönish et al., 1990), each of which could initiate several different cellular processes (Harris et al., 1989; Shafer and Atchison, 1991). Activation of muscarinic receptors has been shown to elevate inositol trisphosphate ( $\text{IP}_3$ ) and intracellular  $\text{Ca}^{2+}$  (Vincentini et al., 1985) followed by release of transmitter from intact cells (Rabe et al., 1987).  $\text{IP}_3$ , an



intracellular second messenger, can cause an increase in intracellular free  $\text{Ca}^{2+}$  by releasing it from intracellular stores (Berridge and Irvine, 1984; Stoehr et al., 1986), which contain fairly high levels of  $\text{Ca}^{2+}$  (2 mM) (Clapham, 1995). Though this amount of  $\text{Ca}^{2+}$  once released to the larger volume of the cytosol may not be sufficient to cause exocytosis, another second messenger produced upon muscarinic receptor activation may indirectly help facilitate release. This messenger, diacylglycerol, in combination with elevated intracellular  $\text{Ca}^{2+}$  causes the activation of protein kinase C (Kishimoto et al., 1980), which enhances transmitter secretion (TerBush et al., 1988) possibly by increasing the  $\text{Ca}^{2+}$  sensitivity of the vesicle mobilization and fusion process (Pozzan et al., 1984). It is also thought that a small amount of extracellular  $\text{Ca}^{2+}$  may enter the cell through a receptor-mediated channel activated by the muscarinic receptor (Pozzan et al., 1986; Inoue and Kenimer, 1988). It is likely that all these processes work cooperatively to bring about the stimulated release of catecholamines. Since several events must occur between the initial stimulation with muscarine and the elevation of cytosolic  $\text{Ca}^{2+}$  to a level able to trigger exocytosis, the longer latency observed ( $103 \pm 11$  s) seems logical. After comparing the dynamics of the three types of stimulated release, it appears that the rate-limiting step in the stimulation-secretion process occurs prior to entry of  $\text{Ca}^{2+}$  to the cytoplasm since this is where the three mechanisms differ.

Although the mechanism of release has a dramatic effect on the time course of release, it does not appear to have a significant effect on the average vesicle DA

content. The distributions of vesicular catecholamine content for the three types of stimulation are also qualitatively similar. This suggests that all the mechanisms are affecting the same or very similar vesicular pools which allows evaluation of the latency data without complications caused by sampling from different populations of vesicles. The cube root histograms of vesicle content imply that there is a uniform concentration of catecholamine within the vesicles, with the radii of released vesicles being distributed in a nearly Gaussian fashion.

The latency data collected for the stimulation of the cells with 10 mM ACh ( $34 \pm 11$  s, first response;  $109 \pm 15$  s, second response) corresponds very closely to that observed for stimulation with nicotine ( $37 \pm 5$  s) and muscarine ( $103 \pm 11$  s) separately. This is expected since ACh binds to both nicotinic and muscarinic receptors and so should trigger exocytosis by both mechanisms. It is surprising, however, that the two responses are temporally resolved. The larger average vesicle size observed for the ACh-stimulated release could indicate mobilization of a different population of catecholamine-containing vesicles. More likely this is due to the slightly older age (10-11 days) of the cells used in comparison to the age of those used in other experiments (7-10 days).

From the data presented here, it can be concluded that although the vesicular pool undergoing exocytosis is unchanged by the mechanism of release, the latency between stimulation and secretion is significantly affected. This suggests that

exocytosis stimulated by mechanisms of increasing degrees of complexity exhibit longer latencies between stimulation and secretion. It appears that all three mechanisms studied here require  $\text{Ca}^{2+}$  for exocytosis and the differences in their time courses can be attributed to the steps prior to the point where  $\text{Ca}^{2+}$  levels have reached the threshold required for DA release. The observed differences in latency could possibly play an important role in intercellular communication by providing another level of discrimination in the processing of neuronal signals.

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## FIGURE LEGENDS

**Figure 1.** Current-Time Traces for Exocytosis at Single PC12 Cells. A 6 s ejection of stimulant (105 mM  $K^+$  (A); 1 mM nicotine (B); 1mM muscarine (C)) from a microinjector was administered at each arrow. The resulting current transients correspond to the oxidation of DA at the electrode tip as it is released from the cell. Detection was performed in the amperometric mode at 650 mV vs. SSCE.

**Figure 2.** Distributions of Latencies Observed Between Stimulation and Secretion. The time between the application of the stimulus and the detection of released DA for each exocytosis event in the first 40 s of release is plotted vs. the percent of the total number of events observed for each type of stimulus. The differences in the time courses of 105mM  $K^+$  (■)(n=17cells; 475 events), 1 mM nicotine (▣)(n=16 cells; 232 events) and 1 mM muscarine (□)(n=19 cells; 439 events) stimulated release are apparent.

**Figure 3. Distributions of the Amount of Catecholamine Released Following Chemical Stimulation of Single PC12 Cells.** The area under each current transient observed in the first 40 s of (A) 105 mM K<sup>+</sup>, (B) 1mM nicotine and (C) 1 mM muscarine stimulated release was converted into moles of DA detected per vesicle using Faraday's law ( $Q = nNF$ , see methods). The total number of moles of DA detected for each exocytosis event was collected into bins having increments of 20 zmol and plotted as the percent of the total number of release events observed. One zmol is  $10^{-21}$  moles.

**Figure 4. Distribution of Vesicle Content for Chemically Stimulated Exocytosis Plotted as the Cube Root of Catecholamine Released.** Plots of the percent of total events detected in the first 40 s of release vs. the cube root of the amount of catecholamine released upon (A) 105 mM K<sup>+</sup> (n=17; 475 events), 1 mM nicotine (n=16; 232 events) and 1 mM muscarine (n=19; 439 events) stimulation. Using the assumption that the catecholamine concentration within each vesicle remains constant, these histograms portray the distribution of vesicle radii.

**Figure 5.** Current-Time Transients for ACh-Stimulated Release from a PC12 Cell. A 6 s ejection of 10 mM ACh from a microinjector was applied at the arrow. The current transients correspond to the DA oxidation at the electrode tip as it is released from the cell. A two phase response is seen corresponding roughly to the time scale of muscarine- and nicotine-stimulated release simultaneously at the same cell. Detection was performed at 650 mV vs. SSCE.

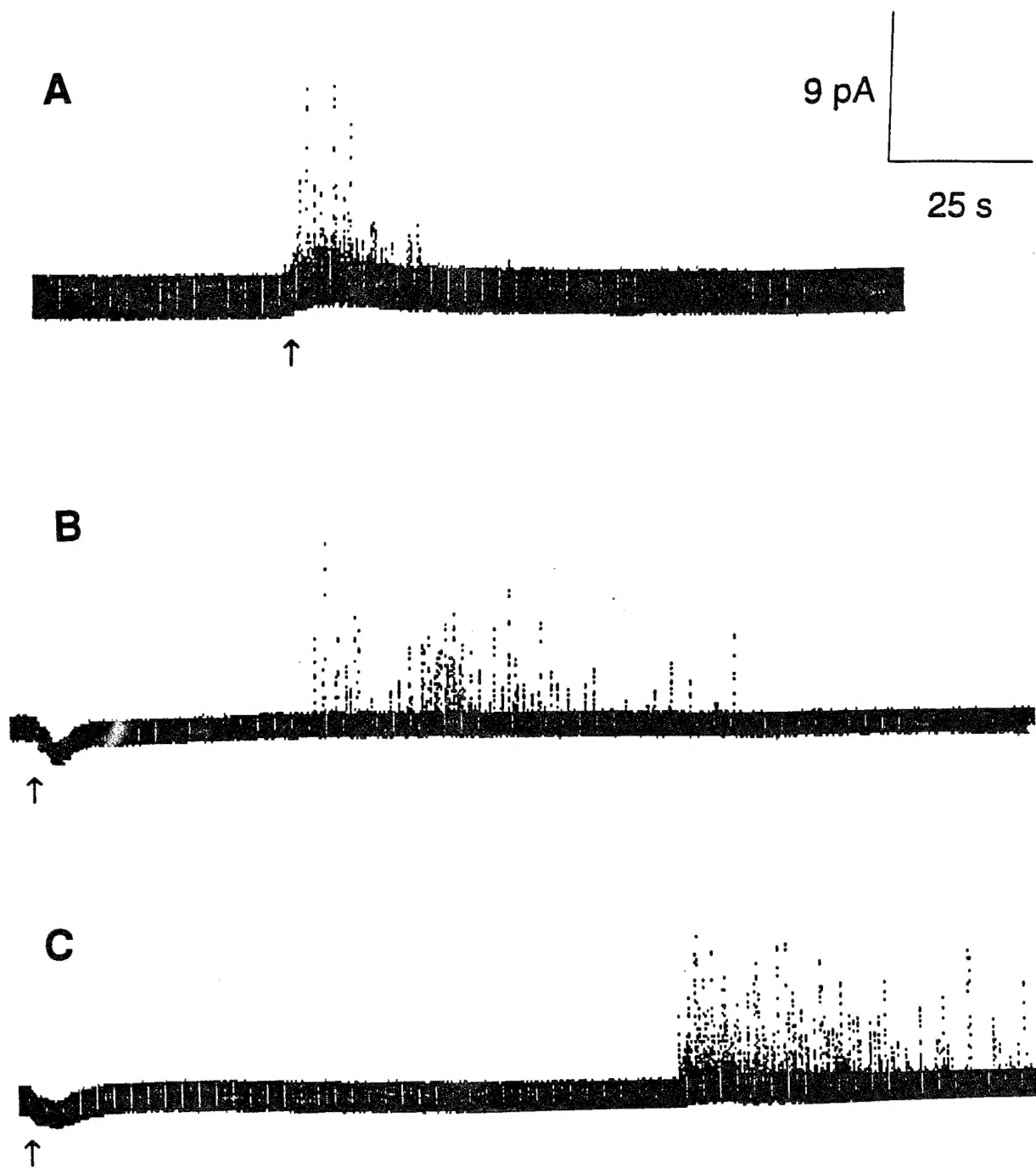


Figure 1

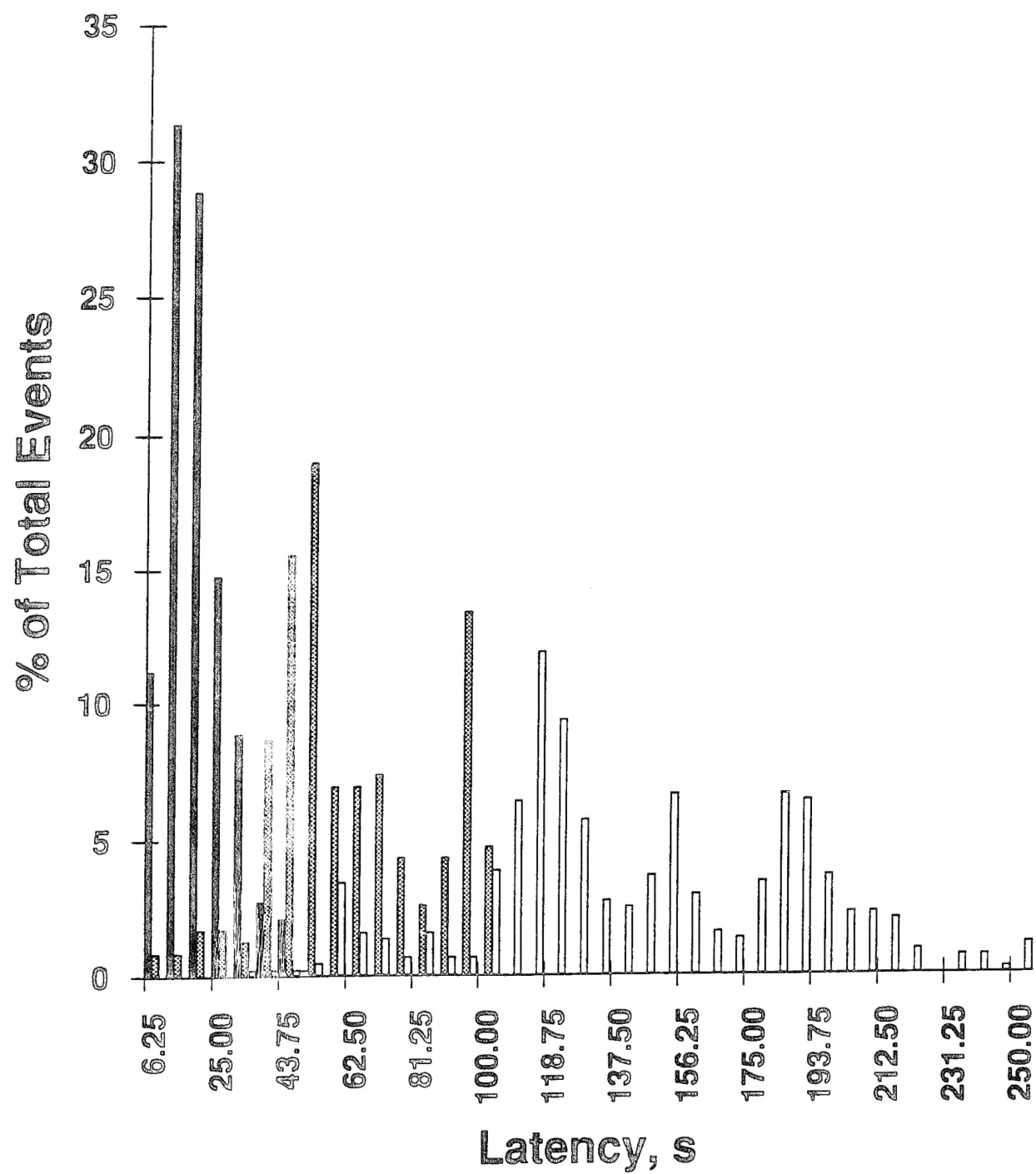


Figure 2

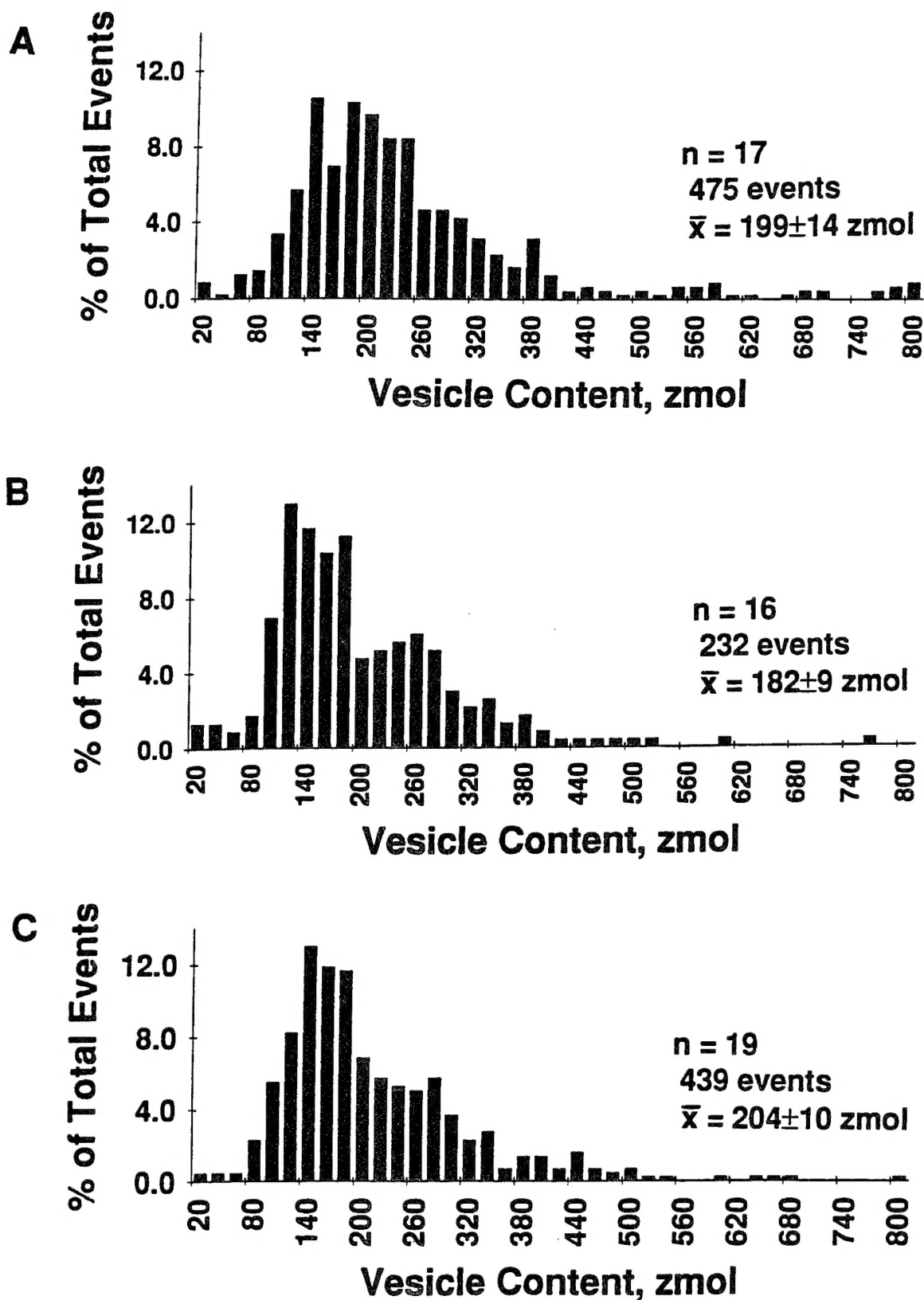


Figure 3



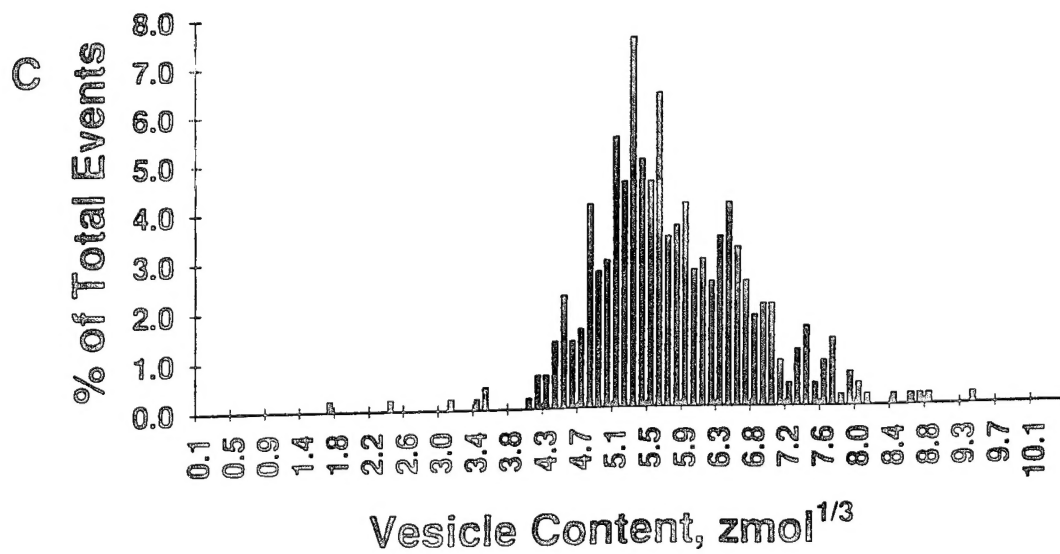
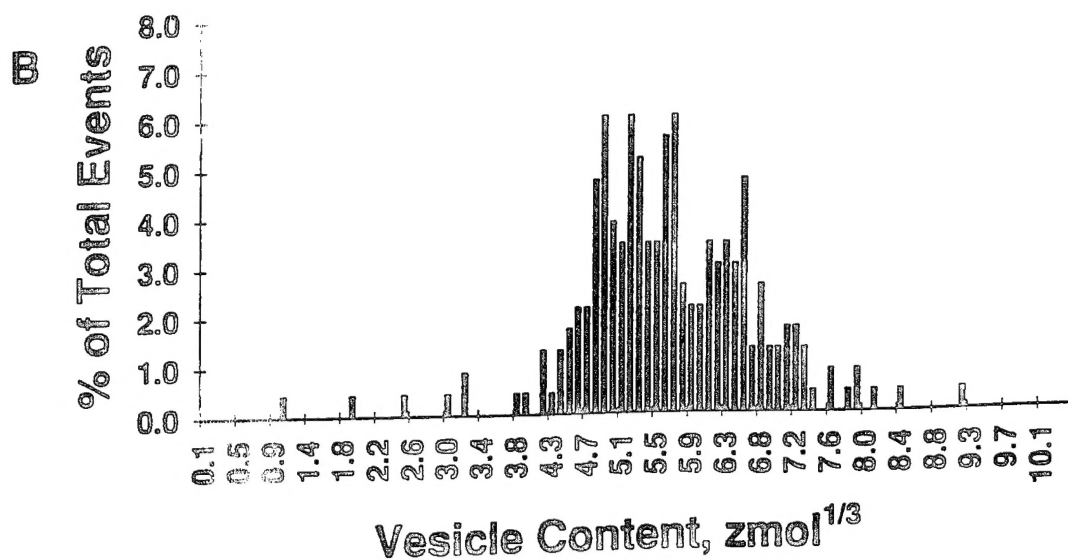
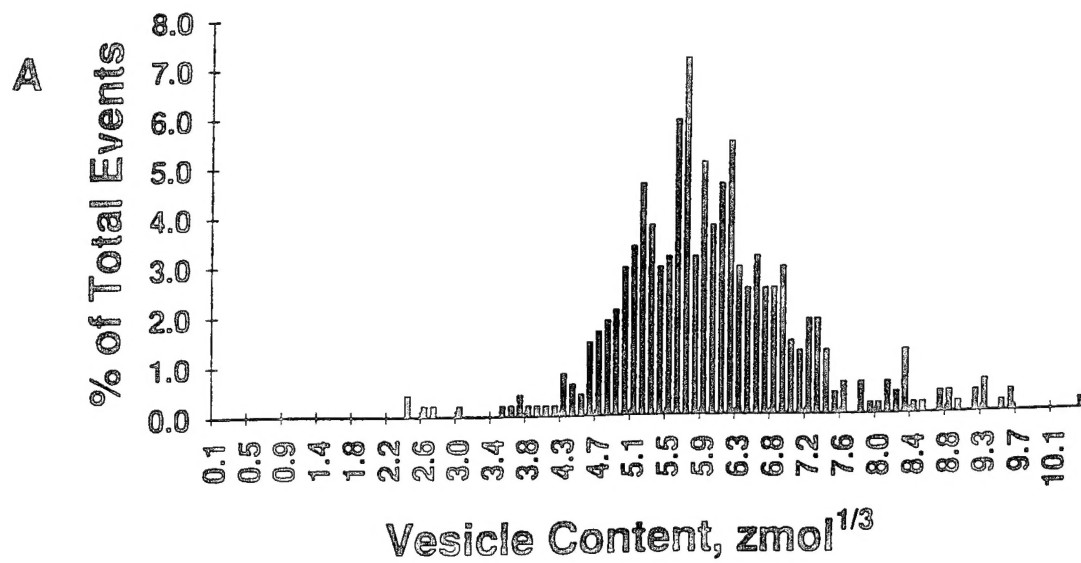


Figure 4